Direct genetic selection for a specific RNA – protein interaction

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ABSTRACT

The decision between lytic and lysogenic development of temperate DNA bacteriophages is determined largely by transcriptional regulation through DNA-binding proteins. To determine whether a heterologous RNA-binding activity could control the developmental fate of a DNA bacteriophage, a derivative of P22 was constructed in which the chosen developmental pathway is regulated by an RNA-binding molecule interacting with its RNA target site located in a phage mRNA. In the example presented, lysogenic development of the phage relies upon R17 coat protein expression in the susceptible host cell and the availability of a suitable coat protein binding site encoded by the phage genome. Through the analysis of phage mutants that are able to grow lytically in susceptible cells that express the coat protein, additional insights were obtained regarding the specific interaction of the R17 coat protein with its RNA binding site. This study also suggests a novel and extremely sensitive strategy for selecting RNA-binding activities *in vivo*.

INTRODUCTION

RNA molecules perform a variety of important and diverse functions for viruses and in cells. To understand how RNA molecules perform their intended function, a description of their structure and their interaction with other macromolecules is necessary. To date, detailed studies of biomolecule-RNA interactions have been mainly limited to *in vitro* methods with purified RNA-binding molecules and RNA substrates (1–7).

Here we describe a sensitive, convenient, and quantitative method to select for a specific biomolecule-RNA interaction in an *in vivo* context. The method employs DNA temperate phages that infect bacteria. Typical members of this family include bacteriophage lambda, which infects *Escherichia coli*, and bacteriophage P22, which infects *Salmonella typhimurium*. The temperate bacteriophages were chosen for this purpose because they continue to offer several significant advantages over other *in vivo* approaches, the most significant of which is the ease

whereby strong genetic selections can be applied in the particular system of interest.

For the P22 DNA bacteriophage, two sets of genes determine the developmental fate of the bacteriophage in an infected host cell (Figure 1a). These genes reside in two genetically defined loci within the bacteriophage genome termed the immunity regions, *immC* and *immI*. The expression of the *c2* gene, which resides in *immC*, determines whether the bacteriophage establishes and maintains a dormant, lysogenic state in the infected host cell. The *c2* protein represses lytic gene functions by binding to two DNA operator regions, termed O_R and O_L (8). Two additional repressor proteins, termed Mnt and Arc, and an antirepressor protein, called Ant reside in *immI*. Expression of these latter three genes occurs from two divergently transcribed operons. One transcription unit consists of P_{mnt} and the *mnt* gene. The second unit consists of P_{ant} and the *arc* and *ant* genes (8). The Mnt and Arc proteins selectively repress *ant* gene expression by binding to two different DNA operator sequences overlapping O_{mnt} (9–11). During the early phase of infection, P_{ant} is utilized, and a burst of Arc and Ant synthesis results. As Arc protein accumulates, it represses transcription from P_{ant} by binding to an overlapping operator site, O_{arc} . Although Arc also represses its own expression through binding O_{arc} , transcription of *mnt* from P_{mnt} is stimulated, leading to the synthesis of Mnt protein. Mnt binds to O_{mnt} to prevent further expression from P_{ant} so that a stable lysogen can form. In addition, Mnt binding to O_{mnt} activates transcription from P_{mnt} (12).

Repression of *ant* gene expression by Mnt is critical for maintaining the lysogenic state because Ant is able to inactivate *c2* repressor function by binding non-covalently to the protein (13). Without functional *c2* repressor, lytic gene functions would be fully expressed, and the lytic growth phase would ensue. Although *ant* gene expression does not influence the developmental fate in the wild-type P22 context during normal infection (8), phages that contain an *arc*(Am) mutation dramatically overproduce Ant after infection (9), which can lead to lytic gene functions being expressed. The *arc*(Am)-bacteriophage can form lysogens, however, if transcription of *ant* is prevented. Thus, the relative amounts of *c2* and Ant proteins

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synthesized in an infected cell will dictate whether an *arc*(Am)-bacteriophage becomes a lysogen or a lytic phage.

Challenge phages are modified versions of P22 in which regulatory events that control the expression of *ant* determine the developmental fate of the phage. They are designed to detect sequence-specific DNA-binding activities using derivatives of P22 that contain DNA target sequences substituted for *O_{mut}* (14). Infection of susceptible cells that express sufficient levels of the cognate DNA-binding protein that binds to the substituted operator results in transcriptional repression of Ant biosynthesis. Because gene expression can be controlled at points beyond transcription initiation, we reasoned that we may be able to modify the original design of the DNA challenge phage to select for RNA-binding activities *in vivo*, as shown in Figure 1b. A P22 derivative that exploits the RNA-binding properties of the coliphage R17 coat protein was constructed to test this hypothesis.

MATERIALS AND METHODS

Description of plasmids and bacteriophages

Construction of R17 coat gene expression plasmids. A 975 bp *Pvu* II fragment containing the RNA bacteriophage R17 coat gene and accompanying 5' flanking region was excised from pTCT5 (a gift from O.C. Uhlenbeck) and inserted into the *Hind*III site of pCKR101(15) using standard recombinant DNA methods (16). The plasmid designated pR17coat(+) contains the R17 coat gene in the same transcriptional sense as the P_{tac} promoter (Figure 2). Deletions within the 5' flanking region sequence that separates the AUG of the coat gene from the P_{tac} promoter were created by digesting pR17coat(+) either with *Sna*BI restriction endonuclease or with the combination of *Sna*BI and *Xba*I restriction endonucleases. The plasmid clone designated as pR17coat(+)ΔS contains approximately 40 bp of heterologous sequences 3' of the P_{tac} promoter and 94 bp of natural R17 flanking sequences 5' to the R17 coat gene AUG start codon (Figure 2). The plasmid clone designated as pR17coat(+)ΔSX contains approximately 40 bp of heterologous sequences 3' of the P_{tac} promoter and 30 bp of natural R17 flanking sequences 5' to the R17 coat gene AUG start codon (Figure 2). One frame-shift mutation was created in the open reading frame for the R17 coat gene. The plasmid pR17coat(+)ΔS was digested with *Sal*I restriction endonuclease, which cleaves the R17 coat gene at the eleventh codon. The *Sal*I termini were made flush with T_4 DNA polymerase, and the plasmid DNA molecule was recircularized with T_4 DNA ligase. The desired clone was characterized by restriction endonuclease digestion using *Pvu*I restriction endonuclease (Gibco/BRL), as ligation of the flushed *Sal*I termini generates this restriction enzyme recognition site. The resulting plasmid, designated pR17coat(+)ΔS/FS13, contains a coat gene mutation in which the (−1) open reading frame is used following the twelfth codon position and terminates with an *ochre* (UAA) codon 91 nucleotides 3' of the frame-shift site (Figure 2).

Construction of the P22_{R17} bacteriophage. The plasmid pPY190 (a gift of Dr. K. Hughes) is a pBR322-based plasmid that carries the P22 *mnt* gene, *P_{arc}*, a mutated *O_{mnt}* site [in which the natural *O_{mnt}* sequence is replaced by *Sma*I and *Eco*RI sites (17)], and the 5' end of the *arc* (*arc'*) gene. This plasmid construct can be used as a vector to introduce cloned DNA binding sites into the phage by homologous recombination (18). For the purposes of installing novel RNA binding sites into the challenge phage context, it was necessary to construct a derivative of pPY190

Figure 1. a. Life cycle of bacteriophage P22. Solid lines refer to expression of regulatory gene products. Dashed lines refer to negative regulatory pathways. For a more detailed discussion, see description in the text. **b.** RNA-dependent challenge phage design. The modified P22 phage contains an amber mutation in *arc* [*arc*(Am)] and a Kn9 cassette inserted into the *mnt* gene (*mnt*::Kn9). The region encoding the mRNA that contains the ribosome binding site of the *ant* gene is substituted by a sequence encoding an RNA sequence (shown by the lollipop) recognized by an RNA-binding molecule. The RNA binding molecule is expressed from an expression vector. The relative amounts of c2 and Ant proteins synthesized in an infected cell will dictate whether the phage becomes a lysogen or undergoes lytic growth. Prior to infection with the phage, the inducer, IPTG was added to the recipient cells. If a sufficient amount of the RNA binding molecule is present in the cell, it will saturate its specific RNA binding site on the newly synthesized *ant* mRNA transcripts following infection and inhibit Ant translation. Repression of Ant synthesis allows c2-mediated establishment of lysogeny. This results in formation of a kanamycin-resistant lysogen.

that contains *ant* gene sequences in addition to the P22 sequences already present. The truncated *arc'* gene in pPY190 is fused to the vector by the unique *Hind*III site in *arc*. The adjacent *Hind*III fragment that carries the remainder of *arc*, the ribosome binding site of *ant*, and the first 100 bp of *ant* was excised from P22 and inserted into pPY190 to create pMMW20. The plasmid pMMW21 is a derivative of pMMW20 in which the ribosome binding site of *ant* is replaced by the DNA encoding the RNA hairpin containing the replicase ribosome binding site of coliphage R17. The construction does not change the coding sequence of *ant* but does alter the 18 bp immediately upstream of the *ant* ATG start codon. The replacement was accomplished using the Kunkel method (19, 20). In brief, a synthetic oligodeoxyribonucleotide (5'-GGCTTCGGTTGTCAGTAAATTGATCATGAGGATTA CCCATGAATAGTATAGCAA-3') was used as a primer to initiate DNA synthesis *in vitro* from a complementary single-stranded phagmid pMMW20 template that contained deoxyuridine residues. A modified version of the original Kunkel procedure was used (21), and the presence of the desired insert was verified by DNA sequence analysis (16).

Figure 2. Plasmids that express the R17 coat protein. The pR17coat(+) contains the R17 coat gene in the same transcriptional sense as the P_{tac} promoter; the pR17coat(+) Δ S contains approximately 40 bp of heterologous sequences 3' of the P_{tac} promoter and 94 bp of natural R17 flanking sequences 5' to the R17 coat gene AUG start codon; pR17coat(+) Δ SX contains approximately 40 bp of heterologous sequences 3' of the P_{tac} promoter and 30 bp of natural R17 flanking sequences 5' to the R17 coat gene AUG start codon; pR17coat(+) Δ S/FS13 contains a mutation in the coat gene which results in the (-1) open reading frame being used following the twelfth codon position and terminates with an ochre (UAA) codon 51 nucleotides 3' of the frame-shift site. The symbols for the restriction enzymes are indicated as *Sma*BI (S), *Xba*I (X), and *Hind*III (H). In all plasmids, the R17 coat gene is designated by the open box that contains an arrow to indicate the orientation of the coat gene open reading frame relative to the transcriptional sense of the P_{tac} promoter.

The R17 ribosome binding site was introduced onto a P22 derivative by homologous recombination in the following fashion. *S. typhimurium* strain MS1883 [*leuA414* Fels⁻ r⁻m⁺ supE40] that contained pMMW21 was infected with P22 *mnt::*Kn9 *arcH1605*(Am) at a multiplicity of infection (MOI) of 10. The mixture was incubated at room temperature for 20 min and 5 ml of LB, which contained 1×E Medium and 0.2% dextrose, was added. The infected cells were incubated with aeration at 37°C until lysis (about 2.5 hr). Dilutions of the lysate were incubated with exponentially growing *S. typhimurium* strain MS1582 [*leuA414* (Am) Fels⁻ supE40 attP::[P22 *sieA44* 16(Am)*H1455* Tprf49]] cells. Top agar was added, and the mixture was plated onto LB plates and incubated overnight at 37°C. The prophage in MS1582 expresses Mnt and c2 so that parental P22 *mnt::*Kn9 *arcH1605*(Am) phage cannot form plaques because Mnt represses O_{mnt} of the phage. The *arc*⁺ recombinant phage (P22 *mnt::*Kn9 $O_{mnt::$ *SmaI-EcoRI arc*⁺), which result from recombination of P22 *mnt::*Kn9 *arcH1605*(Am) with pMMW21, contains the *SmaI* and *EcoRI* sites in place of O_{mnt} and can form plaques because the O_{mnt} site is lost. Phages that acquire the *arc*⁺ gene via recombination with pMMW21 also form turbid plaques because Arc causes partial repression of P_{ant} (see Figure 3a). Turbid plaques were purified twice on MS1582 and twice on MS1883. Depending upon the position of the crossover in the *arc-ant* region, the phages will contain either the wild-type *ant* ribosome binding site or the substituted R17 replicase ribosome binding site (see Figure 3a).

Lysates were prepared from 6 *arc*⁺ phages derived from the described plasmid-phage crosses. In addition, a lysate of P22

*mnt::*Kn9 $O_{mnt::$ *SmaI-EcoRI arcH1605*(Am)*ant::lacZ* was prepared. Because the *lacZ* substitution in the latter phage creates a deletion of gene 9, which encodes the tail fiber protein, the tail fiber proteins were added to the lysate. Exponentially growing MS1883 cells were infected with each of the *arc*⁺ derivatives simultaneously with the P22 *mnt::*Kn9 $O_{mnt::$ *SmaI-EcoRI arcH1605*(Am)*ant::lacZ* phage at an MOI of 5. Single infections were performed as controls. The infected cells were incubated with aeration at 37°C until lysis (about 2.5 hr). The lysates were then treated with CHCl₃ and dilutions were plated on MS1883. Clear, colorless plaques arising from this cross on LB plates supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) contain phages where the *arcH1605*(Am) allele from the P22 *mnt::*Kn9 $O_{mnt::$ *SmaI-EcoRI arcH1605*(Am)*ant::lacZ* phage replaced the *arc*⁺ allele of the artner phage. The P22 *mnt::*Kn9 $O_{mnt::$ *SmaI-EcoRI arcH1605*(Am) *ant::lacZ* parental phage cannot form plaques in the absence of added tail fibers. Clear plaques appeared at a frequency of about 1%. No clear plaques appeared in the mock control crosses in which the *lacZ* fusion phage is omitted. Clear plaque recombinants were purified from each cross on MS1883 and phage DNA minipreps were made. The presence of the substituted R17 sequence as well as the *arc*(Am) mutation were confirmed by direct sequence analysis of recombinant phages. One isolate, designated P22_{R17}, contained the desired R17 replicase gene ribosome binding site fused to the *ant* gene (Figure 3b).

In vivo binding of the R17 coat protein to the R17 replicase gene ribosome binding site in the P22_{R17} bacteriophage

The bacteriophages P22_{R17} and P22₁₄₃ (isogenic to P22_{R17} except that it contains the wild-type ribosome binding site of *ant*) were used for infections of *S. typhimurium* strain MS1868 [*leuA414*(Am) Fels⁻ r⁻m⁺] cells containing either pR17coat(+), pR17coat(+) Δ S, pR17coat(+) Δ SX. The strains to be infected were grown overnight at 37°C in LB broth supplemented with ampicillin (30 μg/ml). The cells were then subcultured 1:50 into the same medium and grown until the culture density was about 5×10⁸ cells/ml. The cells were subcultured 1:4 into LB-ampicillin medium or LB-ampicillin medium supplemented with isopropyl-β-D-thiogalactopyranoside (IPTG) at the following concentrations: 0.01 mM, 0.1 mM, 0.5 mM, and 1 mM. After an additional hour of incubation at 37°C, the culture was infected with the challenge phage at an MOI of 20 to 25. After incubation at room temperature for one hour, aliquots of uninfected cells were serially diluted and plated on LB agar plates supplemented with 30 μg/ml ampicillin and with IPTG at the same concentration used in the previous induction to determine the number of viable cells. To measure the number of lysogens formed following infection of the cells with the challenge phage, aliquots of infected cells were serially diluted and plated on LB plates supplemented with 30 μg/ml ampicillin, IPTG at the same concentration used in the previous induction, and 40 μg/ml kanamycin. The frequency of lysogenization (expressed as % lysogeny at a given IPTG concentration) was calculated as the number of colonies obtained on the LB plates containing ampicillin and kanamycin divided by the number of viable colonies obtained on the LB plates containing ampicillin multiplied by 100.

Mutagenesis of the P22_{R17} bacteriophage

The mutagenesis method was used as described (22). Briefly, pR17coat(+) was introduced into *S. typhimurium* strain JG1201

Figure 3. Construction of the P22_{R17} bacteriophage. **a.** Homologous recombination between the *imm1* regions of P22 *mnt::Kn9 arch1605(Am)* (top) and pMMW21 (middle) yields two types of recombinant phages containing the *SmaI-EcoRI* sites and the *arc⁺* gene of pMMW21. Crossovers at A and 1 yield recombinant phages that contain the wild-type *ant* ribosome binding site (bottom left). Crossovers at A and 2 yield recombinant phages that contain the pMMW21 *SmaI-EcoRI* sites and the R17 ribosome binding site adjacent to *ant* (bottom right). The R17 ribosome binding site on pMMW21 is designated by the lollipop. **b.** Recombination of the *arch1605(Am)* mutation into the *arc⁺* derivative of P22 *mnt::Kn9 O_{mnt::SmaI-EcoRI}* containing the R17-substituted ribosome binding site. P22 *mnt::Kn9 arch1605(Am) ant::lacZ* contains the *arch1605(Am)* mutation and a *lacZ* fusion to the 5' end of *ant*. The *arch1605(Am)* recombinant derivatives of P22 *mnt::Kn9 O_{mnt::SmaI-EcoRI arc⁺}* containing the R17-substituted ribosome binding site, arise by homologous recombination between the parental phages in mixed infections. A crossover between the *arch1605(Am)* site and the substituted R17 replicase gene ribosome binding site (region 1) produces the desired recombinant, P22_{R17} (bottom left). Recombination in *ant* DNA to the right of the R17 replicase gene ribosome binding site (region 2) is rare because of the small amount of DNA homology that is shared between the two phages in that region.

(TH564/pMUC12; TH564 genotype: *leuA414(Am) Fels⁻ supE40 attP::[P22 sieA44 Δ (*mnt-a1* TnI) Amp^{s9} - Tpf184]*) by electroporation. P22_{R17} (1×10^9 pfu/ml) was irradiated with ultraviolet light, and 0.1 ml of dilutions were incubated with 0.1 ml log phase JG1201/pR17coat(+) cells which had been previously induced 60 min for R17 coat protein expression with IPTG at a concentration of 0.01mM. After allowing for phage adsorption, the mixtures were plated in 2.5 ml top agar onto LB agar plates supplemented with ampicillin and IPTG. The plates were incubated at 37 °C overnight. Clear plaques were purified on JG1202/pR17coat(+) cells that were grown in LB medium supplemented with ampicillin and IPTG and plating on LB agar

plates containing ampicillin and IPTG. High titer phage lysates were made using MS1883 as the host.

DNA sequence analysis of P22_{R17} wild-type and mutant phages

DNA from isolated plaques was first amplified by symmetric PCR amplification and single-stranded DNA template was generated with asymmetric PCR amplification as modified (23). The oligonucleotide that was used as a primer in DNA sequence analyses (5'-GCGGTAAGAACATGCTGTC-3') anneals to *ant* gene DNA and primes DNA synthesis toward the region containing the ribosome binding site. DNA sequencing was

Figure 4. *In vivo* binding of R17 coat protein to the R17 replicase gene ribosome binding site in the P22_{R17} bacteriophage. MS1868 cells that contain pR17coat (+) (□), pR17coat (+) ΔS (○), or pR17coat (+) ΔSX (△) were grown at the indicated IPTG concentrations and infected subsequently with P22_{R17}. The frequency of lysogenization (expressed as % lysogeny at a given IPTG concentration) was calculated as described in Materials and Methods.

performed with the T₇ DNA sequencing kit ('Sequenase' United States Biochemical Corp.).

RESULTS

P22_{R17} bacteriophage lysogen formation directed by the R17 coat protein

In an effort to develop a bacteriophage model system to study RNA-protein interactions *in vivo*, a P22 derivative (P22_{R17}) that exploits the RNA-binding properties of the coliphage R17 coat protein was constructed as shown in Figure 3. The bacteriophage R17 is a single-stranded RNA phage that infects *Escherichia coli*. The coat protein serves dual roles during R17 infection. It is both the primary capsid protein and the translational repressor of the phage's replicase gene. As coat protein accumulates within the infected cell, it inhibits translation of the replicase protein by binding to an RNA hairpin that contains the ribosome binding site in the replicase mRNA (24). In the recombinant bacteriophage P22_{R17}, the ribosome binding site of *ant* has been replaced with sequences encoding the RNA hairpin of the phage R17 replicase ribosome binding site (Figure 3). Infection of cells with P22_{R17} forms clear plaques, indicating that Ant is translated efficiently from the modified *ant* mRNA (not shown).

Different results are obtained with P22_{R17} infection of cells that express the R17 coat protein. For this experiment, we constructed plasmids that express the R17 coat gene under control of the P_{lac} promoter (Figure 2). The plasmids also contain a copy of *lacI^q* so that the levels of coat protein can be controlled by the inducer, IPTG. Infection of cells that contain the coat gene and a substantial portion of untranslated region sequence [from pR17coat(+)] with the P22_{R17} phage resulted in lysogens at a frequency of about 30% at all concentrations of IPTG (Figure 4).

Figure 5. Frequency of lysogenization obtained for P22₁₄₋₃, P22_{R17} and mutant P22_{R17} phages following infection of MS1868 cells that express different constructs of the R17 coat gene. The downward arrows represent instances in which no ampicillin/kanamycin-resistant lysogens were detected following the plating of an undiluted aliquot of the infected cell suspension, so the indicated value is an upper limit. The indicated MS1868 strains were treated with IPTG at a final concentration of 0.75 mM prior to infection with the different challenge phages. The phage infection experiments and the frequency of lysogenization calculations (expressed as % lysogeny) were performed as described in Materials and Methods except for two differences in experimental protocol. First, the viable cells were determined from plating uninfected cells on LB plates containing 100 μg/ml ampicillin and 0.75 mM IPTG. Second, the infected cells were plated onto LB plates containing 100 μg/ml ampicillin, 0.75 mM IPTG, and 50 μg/ml kanamycin.

In a similar experiment, no lysogens were formed following P22_{R17} infection of cells containing only the vector sequences (not shown). P22_{R17} infection of cells that contain plasmids that encode the coat gene with progressively shorter portions of 5'-untranslated region sequence present [in pR17coat(+)] did not yield viable lysogens at low IPTG concentrations (< 0.01mM; Figure 4). At higher IPTG concentrations (0.1mM to 1mM; Figure 4), P22_{R17}-infected cells that contain pR17coat(+)] yielded lysogens at the same efficiency as found for P22_{R17}-infected cells that contain pR17coat(+).

Control experiments demonstrated that the efficient lysogenization by P22_{R17} in cells producing R17 coat protein depends upon the type of ribosome binding site located adjacent to the *ant* open reading frame. For example, a P22 phage (P22₁₄₋₃) with the wild-type *ant* ribosome binding site did not form lysogens in the MS1868 host producing R17 coat protein, because these cells produce no repressor that can inhibit *ant*

Table I. Proposed structure of the substituted R17 ribosome binding site in P22_{R17} and in phage mutants

	wild-type	A(-4)G	A(-10)U	C(-1)A	G(-8)A	G(-9)C	U(-12)C
A	<pre> U U A-C A-4 G-C -10 A- G-C -1 U-A +1 A-U C-G U-A UGA AU </pre>	<pre> U U A-C G G-C A- G-C U-A A-U C-G U-A UGA AU </pre>	<pre> U U A-C A G-C U- G-C U-A A-U C-G U-A UGA AU </pre>	<pre> U U A-C A G-C A- G-C A U-A A-U C-G U-A UGA AU </pre>	<pre> U U A-C A G-C A- G-C U-A A-U C-G U-A UGA AU </pre>	<pre> U U A-C A G-C A- G-C U-A A-U C-G U-A UGA AU </pre>	<pre> U U A-C A G-C A- G-C U-A A-U C-G U-A UGA AU </pre>
B	<pre> U U A-C A G-C A- G-C U-A A-U C-G A-U_P HO A A </pre>	<pre> U U A-C G G-C A- G-C U-A A-U C-G A-U_P HO A A </pre>	<pre> U U A-C A G-C C- G-C U-A A-U C-G A-U_P HO A A </pre>	<pre> U U A-C A G-C A- G-C U-A A-U C-G A-U_P HO A A </pre>	<pre> U U A-C A G-C A- G-C U-A A-U C-G A-U_P HO A A </pre>	<pre> U U A-C A G-C A- G-C U-A A-U C-G A-U_P HO A A </pre>	<pre> U U A-C A G-C A- G-C U-A A-U C-G A-U_P HO A A </pre>
Ka:	$3 \times 10^8 \text{ M}^{-1}$	$< 10^5 \text{ M}^{-1}$	$< 10^5 \text{ M}^{-1}$	$\sim 10^5 \text{ M}^{-1}$	$2 \times 10^7 \text{ M}^{-1}$	$< 10^5 \text{ M}^{-1}$	$< 10^5 \text{ M}^{-1}$
Effect on Structure:		Loop A(-4) Alteration	Bulged A(-10) Alteration	Lower Stem Disruption	Lower Stem Disruption	Upper Stem Disruption	Lower Stem Disruption
Ref.	2, 4	2	4	2	2	2	4

A. Nucleotide sequence of the region containing the R17 replicase gene ribosome binding site in P22_{R17} and in phage mutants.

B. Nucleotide sequence and thermodynamic binding constants for R17 Coat protein binding of natural and mutant R17 replicase gene ribosome binding site synthetic RNA oligonucleotides.

expression (Figure 5). A mutant gene for coat protein that contains a frame-shift mutation [Figure 2, pR17coat(+) Δ S/FS13] also did not promote the formation of lysogens above the background level (Figure 5).

Phage-encoded mutations that allow lytic growth of P22_{R17} in cells containing the R17 coat protein have alterations in the coat protein binding site

The results discussed in the previous section suggest that R17 coat protein binds to the RNA hairpin structure present on the modified *ant* mRNA transcript of P22_{R17}. A key prediction of this interpretation is that mutations in P22_{R17} phage that acquire the ability to produce Ant after infection of cells containing pR17coat(+) should contain alterations in the RNA hairpin structure containing the ribosome binding site. To test this prediction, the P22_{R17} phage was irradiated with short wavelength ultraviolet light, and phage mutants were selected that overcome repression by the coat protein (Table I). Because these phage mutants grow lytically in cells that express the coat protein, they form clear plaques. Sequencing of these mutant phages revealed mutations predicted to change the RNA hairpin structure recognized by coat protein (Table IA).

To assess whether any of these P22_{R17} mutant phages form lysogens in cells that produce R17 coat protein, exponentially growing MS1868/pR17coat(+) hosts were infected with each of the mutant phages, and the frequencies of lysogenization were

determined. With the exception of P22_{R17}[A(-10)U], none of the P22_{R17} mutant phages formed lysogens. The low level of lysogenization obtained with P22_{R17}[A(-10)U] suggests that the R17 coat protein binds weakly to the altered RNA hairpin structure, resulting in partial repression of Ant protein translation.

DISCUSSION

In this manuscript, we demonstrate a method to analyze specific RNA-binding activities *in vivo* that offers significant advantages over traditional reporter gene screening methods. The described bacteriophage system offers two selection schemes that enable the investigator to discern productive and non-productive binding interactions between the RNA-binding molecule and the RNA target site. Productive RNA-binding interactions result in the repression of Ant protein biosynthesis; thus, lysogen formation can be used to select for these interactions. Non-productive RNA-binding interactions allow for the production of Ant protein and the expression of lytic cycle functions. In this latter context, the described method is ideal for selecting loss of the RNA-protein interaction. The extent to which the Ant protein is expressed (or c2 repressor function persists) can be assessed by the number of colonies that grow on LB plates containing kanamycin and by the frequency of clear plaque formation on a lawn of susceptible bacteria. The dual selection scheme afforded by the phage system, when coupled to the ability to titrate the available

RNA-binding activity molecules, provides a new approach to selecting and characterizing RNA-binding activities by a genetic means.

The described bacteriophage system enabled us to select for several mutant RNA structures that are defective in R17 coat protein binding. Previously, Uhlenbeck and co-workers performed extensive binding experiments with synthetic RNA oligonucleotides *in vitro* (2–4) to identify three classes of RNA changes that lower coat protein binding affinity markedly (Table IB). The regions found to be important for coat protein binding include (i) the identity of the bulged adenosine of the hairpin structure (3, 4), (ii) a well conserved adenosine located in the loop sequence of the hairpin (2), and (iii) the secondary structure of the hairpin stems that flank the bulged adenosine (2, 4). Each of these classes of changes is represented by mutant P22_{R17} phages that overcome repression by the R17 coat protein, which is consistent with the model for recognition of the RNA structure by the protein that was proposed previously (2–4). Specifically, five of the six mutants that were obtained from this mutagenesis experiment represent previously uncharacterized RNA structures defective in coat binding (Table IA). The *ant* mRNAs that contain these mutant R17 coat protein binding sites from these phages are not bound efficiently by the coat protein expressed in cells containing pR17coat(+), as inferred from the low frequency of lysogenization following phage infection (Figure 5). For the P22_{R17}[A(–10)U] mutant phage, some repression of Ant protein translation, though inefficient, apparently provides for a cellular environment whereby c2 repressor function persists and lysogens form at low frequencies (Figure 5).

The precise molecular contacts that are established between the R17 coat protein and the folded RNA structure remain to be elucidated for this translational operator system. It is evident from previous work (2–4), and reiterated in this work, that the folded RNA structure is critical to the stable association of the RNA with the coat protein. Through the analysis of a collection of coat protein mutants that are defective for RNA-binding, Peabody has pinpointed several amino acid residues that contribute to the protein's ability to bind tightly with the wild-type RNA (25). These residues cluster together on three adjacent strands of a β -sheet for each monomer of the coat protein dimer and would be exposed to the RNA, as inferred from the crystal structure of the structurally related MS2 coat protein capsid (26). To address the issue of RNA-binding specificity, it should be possible to employ the bacteriophage system to select for second-site coat protein suppressors that recognize the mutant RNA binding sites by simply selecting for lysogens containing P22_{R17} phages with these mutant sites. Like that which has been described for identifying altered specificity mutants for two DNA-binding proteins (22, 27), the isolation of such suppressors would enable a detailed genetic and biochemical analysis of this particular RNA–protein interaction.

In the present application, a heterologous RNA-binding activity can be selected with modified P22 bacteriophages that contain the RNA target site as part of the *ant* ribosome binding site. Because of specific sequence limitations, structural requirements, or size constraints, RNA target sites for certain RNA–bio-molecule interactions may not be readily incorporated into the context of the ribosome binding site of the *ant* mRNA to enable selection for the desired RNA–biomolecule interaction *in vivo*. We are testing whether specific RNA–protein interactions may be detected with modified P22 phages that contain an RNA target site located adjacent to—as opposed to replacing—the wild-type

ant ribosome binding site. Nevertheless, the selection system that we have described should be applicable to many studies involving the interactions of RNA with other macromolecules.

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